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The Volatile Acid Production of Starters and of Organisms Isolated From Them

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THE VOLATILE ACID PRODUCTION OF STARTERS AND OF ORGANISMS ISOLATED FROM THEM

BY B. W. HAMMER AND D. E. BAILEY

The introduction of starters represents one of the distinct advances made in the field of dairying. Altho starters have been widely used their careful study has been very much neglected and the men who handle them extensively are often unable to explain the results obtained. The increasing use that is being made of starters in the manufacture of butter, various types of cheese, and fermented milk drinks makes it essential that they be studied with the idea of securing information regarding the reasons for the variations that occur in the quality. The present paper gives the results secured in a study of the volatile acid production of starters and of cultures of the bacteria isolated from them.

When the common lactic acid-producing bacteria grow in milk the lactose fermented is generally believed to be changed into (a) lactic acid and (b) additional products, such as organic acids other than lactic, alcohols, esters, etc. From the standpoint of the odor and flavor of soured milk the additional products are considered to be of very great importance, both as regards their quantity and quality; since the lactic acid is odorless, these products are largely responsible for the odor of the sour milk and in addition play an important part in determining the flavor.

The volatile acids constitute an important part of the additional products. While acetic acid is apparently the most important[1], the acids produced undoubtedly vary widely with the organisms used in souring the milk and possibly to a certain extent with the conditions such as temperature, air supply, etc., under which the souring takes place.

METHODS USED.

The determination of the volatile acidity in fermented milk offers some difficulties, altho results that are satisfactory from a comparative standpoint can quite easily be secured. Two general types of methods are available: the one[2] consists of half neutralizing the total acidity with n/10 NaOH, evaporating to dryness on a water bath with frequent stirring and, after treating with 20 c.c. boiling distilled water, again adding n/10 NaOH until the neutral point is reached, the difference between the original acidity and that of the evaporated portion being regarded as acetic acid; the second method consists in titrating the acid distilled, generally with steam, from the milk.

In the work herein reported a 250-gram portion of the fermented milk was distilled with steam, after the addition of 15 c.c.

[1]Evans, Alice C. A Study of the Streptococci Concerned in Cheese Ripening. Jour. Agr. Res. 13. 238. 1918.
[2]Richmond, H. D. Dairy Chemistry, Sec. Ed. 142. 1914.

of approximately n/1 H_2SO_4*, until 1,000 c.c. of distillate was secured after which the distillate was titrated with n/10 NaOH, using phenolphthalein as an indicator. Distilled water was used in the steam can and was allowed to boil for some little time before connecting up and starting the distillation. Commonly 5 g. of Na_2SO_4 (anhydrous) were used in the milk to aid in keeping down the foaming. The results obtained were expressed as the number of c.c. of n/10 alkali required; accordingly, the figure given means the c.c. of n/10 NaOH required for the neutralization of the first liter of distillate obtained when a 250-gram portion of milk was distilled with steam. The method used gave a good agreement of duplicates and was considered to be satisfactory from the standpoint of comparative results. By no means all of the volatile acidity is secured[3] where only 1,000 c.c. of distillate are collected, and it is undoubtedly true that the percentage of the total that is secured is only approximately constant because of the differences in the acids present in the various samples of milk, but it seems that the method is of sufficient accuracy for the work for which it was used, since the main object of most of the distillations was to determine whether there was a high or a low volatile acidity.

The total acidity of the milk was usually determined by titrating 20 g. with n/10 NaOH using phenolphthalein as an indicator; in a few instances smaller amounts of milk were used. In all cases the results were calculated as the percent of lactic acid.

The sterile milk used for cultures was skimmilk sterilized in the autoclave in 325 c.c. portions, using an exposure of from 10 to 15 pounds for from 20 to 30 minutes. Sterilization usually darkened the milk considerably but decreasing the pressure or time sometimes resulted in defective sterilization and loss of the milk. When the sterile uninoculated milk was distilled according to the method outlined above the first 1,000 c.c. of distillate usually required from 1.7 to 2.8 c.c. of n/10 NaOH to neutralize it. Occasionally a lot of sterile milk was secured which required up to 5 c.c. of n/10 NaOH to neutralize the distillate, but such lots were discarded.

The containers used for the milk were for the most part wide-mouthed bottles or flasks stoppered with cotton so that there was presumably a good air supply.

In a few instances pasteurized milk was used. In these cases the pasteurizing exposure was high, being from 80 to 95°C. for from 45 to 75 minutes, in order to eliminate as far as possible the action of organisms other than those inoculated.

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*Evans (Jour. Agr. Res. 13. 238. 1918) decided that when $_2$ O_4 was used small amounts of formic acid were formed during the distillation from the action of the acid on some constituent of the milk, and used phosphoric acid instead.

[3]Dyer, D. C. A New Method of Steam Distillation for the Determination of the Volatile Fatty Acids, etc. Jour. Biol. Chem. 28. 445. 1917.

RESULTS OBTAINED.

The Volatile Acid Production of Good Starters.

In order to get a definite idea of the normal volatile acid production of good starters grown at the usual temperatures, a number of distillations were carried out according to the method outlined; a portion of the results obtained are presented in table I.

From table I it is evident that the volatile acidities of the starters worked with, which were from a number of sources, varied from 31.2 to 37.6 while the total acidities varied from 0.87 to 1.08%. The total acidity of most of the starters was higher than is ordinarily desired for propagating purpose and more nearly represents a point to which the acidity will progress rapidly. There seems to be no difference between the results obtained on sterilized and those obtaind on pasteurized milk. All of the starters had a reasonably good flavor and odor, while a number of them were exceptionally good. The data presented, which have been extensively confirmed, suggest that a volatile acidity approximating 35 is present in a good starter that has been well ripened.

From many distillations, some of which are recorded later, it is quite evident that a low volatile acidity indicates that the starter is lacking in flavor and aroma and possesses none of the delicate character present in a desirable product. Other distillations show that a starter may have a volatile acidity approximating 35 and still have a poor flavor and odor and in general be very undesirable, so that a high volatile acidity is no assurance that the starter yielding it is a satisfactory one.

THE VOLATILE ACID PRODUCTION OF CULTURES OF *BACT. LACTIS ACIDI*.

The volatile acid production of cultures of *Bact. lactis acidi* isolated from good starters, milk and cheese, by the plate method,

TABLE I. VOLATILE ACID PRODUCTION OF GOOD STARTERS GROWN AT THE USUAL TEMPERATURES.

Starter	Type of milk inoculated	Total acidity	Volatile acidity*
B1	Sterilized	.99	31.5
C1	"	.87	31.2
E1	"	1.08	37.6
E2	"	1.06	35.8
E3	"	1.06	35.5
E4	"	1.08	32.8
E5	"	1.08	31.5
E6	"	1.03	33.3
E7	"	.95	35.8
A1	Pasteurized	1.08	33.7
A2	"	1.07	35.2
B2	"	.93	33.7
D1	"	1.01	37.6

*As explained under "Methods Used" these values represent the c. c. of n-10 alkali required for the neutralization of the first liter of distillate obtained when 250 grams of milk were distilled with steam after the addition of 15 c. c. of approximately n-1 H_2SO_4.

was determined in a considerable number of instances in order to compare it with the volatile acidity secured with starters direct. A portion of the data secured are presented in table II; sterile milk was used in all cases and after inoculation was held at 20°C. until the total acidity had practically reached its maximum.

From the results given in table II it is evident that the volatile acidities secured with cultures of *Bact. lactis acidi* isolated from various sources by the plate method were much lower than the volatile acidities secured with good starters. The values reported in table II for the 35 cultures isolated from starters from four different sources varied from 4.3 to 8.8 and averaged 6.6 while the 15 cultures isolated from milk and cheese varied from 4.7 to 18.2, with but few running over 10.0, and averaged 9.2; the average of the volatile acidities for the 50 cultures was 7.4.

A comparison of the total acidities reported in tables I and II shows that, while there was a certain amount of overlapping, the total acidities reported in table I were higher than those reported in table II; the general relationship suggested is that a high volatile acidity is accompanied under the usual conditions by a high total acidity.

Evans[4], who has studied the volatile acid production by streptococci from the standpoint of cheese ripening, states that, "Cultures of *S. lacticus* agree, therefore, in producing a small and fairly constant quantity of acetic acid in milk cultures, equivalent to about 0.12 gm., calculated for 1 liter of milk." In comparing *S. lacticus* with certain other organisms this investigator gives 10.00 c.c. as the quantity of n/10 acetic acid in 500 c.c. of milk.[5]

The results presented in table II can be roughly compared with those of Evans as follows:

Av. volatile acidity per 250 grams of milk with 50 cultures of *Bact. lactis acidi*	7.4 c.c. n/10
Av. volatile acidity secured per 250 grams of unfermented milk	2.2 c.c. n/10
Av. volatile acidity developed by *Bact. lactis acidi* per 250 grams of milk	5.2 c.c. n/10
Av. volatile acidity developed by *Bact. lactis acidi* per 500 grams of milk	10.4 c.c. n/10
Wt. of volatile acid (calc. as acetic) per 1,000 grams of milk	0.1248 grams

From this comparison it is evident that there is a close agreement between the average results secured on the volatile acid production by *Bact. lactis acidi* and the results reported by Evans (*S. lacticus*) and this agreement shows that the volatile acidity values secured by Evans are, like those secured with *Bact. lactis acidi*, much lower than the values obtained when starters are distilled.

[4]See ref. 1. p. 240.
[5]See ref. 1. p. 245.

Because of the failure in early trials to isolate by plating methods, organisms which produce a volatile acidity approximating that secured from starters, an attempt was made to isolate an organism of this type by the use of increasing dilutions. The dilution water in varying amounts was transferred to tubes of litmus milk and the tubes numbered consecutively, beginning with the tube containing the largest amount of the original material. An attempt was made to carry the dilutions high enough so that

TABLE II. VOLATILE ACID PRODUCTION BY CULTURES OF *BACT. LACTIS ACIDI* ISOLATED BY THE PLATE METHOD AND GROWN AT 20°C.

Culture	Source	Total Acidity	Volatile Acidity
1	Starter A	.80	4.5
2	" A	.80	5.1
3	" A	.86	4.3
4	" A	.87	4.5
5	" A	.87	4.5
6	" A	.91	7.0
7	" A	.88	5.3
8	" B	.96	8.6
9	" B	.94	8.8
10	" B	.94	8.3
11	" B	.93	8.8
12	" B	.94	8.3
13	" B	.94	8.5
14	" B	.97	8.3
15	" C	.94	7.3
16	" C	.76	6.7
17	" C	.75	7.1
18	" C	.78	6.5
19	" C	.77	6.9
20	" D	.70	5.6
21	" A	.83	4.3
22	" A	.86	4.6
23	" A	.85	4.3
24	" A	.81	7.9
25	" A	.84	6.7
26	" A	.86	7.0
27	" A	.86	7.4
28	" A	.88	7.1
29	" A	.86	7.3
30	" A	.81	8.7
31	" A	.78	5.7
32	" A	.92	6.4
33	" A	.92	7.5
34	" A	.90	6.3
35	" A	.90	6.4
			Av. 6.6
36	Milk	.74	6.1
37	"	.73	6.9
38	"(heat resistant Bla)	.76	6.4
39	" " " "	.75	6.9
40	"	.95	10.0
41	Cheese	.96	8.2
42	"	.85	15.3
43	"	.88	18.2
44	"	.93	9.5
45	Milk	.95	13.8
46	"	.84	11.7
47	"	.84	4.7
48	"	.94	5.8
49	"	.97	6.4
50	"	.94	8.7
			Av. 9.2
			Gen'l Av. 7.4

some tubes gave no growth. Different tubes showing growth were inoculated into sterile milk and, after holding, the volatile acidity was determined. In the majority of the trials the last tubes to show growth gave a low volatile acidity while others, containing larger amounts of the original material, were found which gave a high volatile acidity. A sufficient portion of the data obtained with the graded dilutions to show the trend of the results are given in table III. Other data secured, but not included in table III, indicate that if the dilutions are not properly graded there will be a failure to secure at the end of the series cultures producing only a low volatile acidity.

While it would be unjustified to assume that only one organism was introduced into the last tube showing growth in each series, this was in all probability true in some cases at least, while in the other the number introduced was very limited; on this assumption the results presented in table III agree with those presented in table II in that limiting the source of the inoculating material to one colony on a plate or one or a few cells existing in milk, results in a low volatile acid production. The falling off in the volatile acid production is not gradual with a decrease in the number of organisms inoculated but occurs suddenly as is shown by dilutions 1, 3, and 4 (table III); in dilution 1 e. g. tube 12 gave a high volatile acidity while tube 13 did not and this same situation is shown in the other dilutions mentioned. The results presented in table III bear out the general relationship between high total acidity and high volatile acidity that has already been pointed out.

TABLE III. THE VOLATILE ACID PRODUCTION BY VARIOUS DILUTIONS OF STARTERS.

Dilution 1			Dilution 2		
Tube No.	Total Acidity	Volatile Acidity	Tube No.	Total Acidity	Volatile Acidity
12	1.04	38.6	1	.94	29.6
13	.95	6.8	2	.94	30.7
			11	.82	4.8
			12	.86	4.4
			13	.82	4.4
			14	.79	4.3

Dilution 3			Dilution 4		
Tube No.	Total Acidity	Volatile Acidity	Tube No.	Total Acidity	Volatile Acidity
1	1.00	29.0	5	.90	20.9*
2	1.00	29.2	6	.98	7.3
13	.86	27.7			
14	.84	5.2			

*Very young culture.

INFLUENCE OF TEMPERATURE ON VOLATILE ACID PRODUCTION.

The influence of temperature on the volatile acid production was studied by carrying starters at 37°C. and at 20°C. and running volatile acidity determinations after varying numbers of transfers. Some of the results secured are presented in table IV.

TABLE IV. INFLUENCE OF TEMPERATURE ON THE VOLATILE ACID PRODUCTION BY STARTERS.

Starters carried at 37°C.			Starters carried at 20°C.		
Generations	Total Acidity	Volatile Acidity	Generations	Total Acidity	Volatile Acidity
10	.74	5.3	8	1.09	34.0
19	.71	6.3	6	1.10	34.0
6	.86	9.8	6	1.07	39.3
10	.90	8.0	18	1.03	35.2
2	.88	7.7	10	1.04	32.6
16	.80	5.9	----	----	----

The results given in table IV show that at 37°C. the volatile acid production was very low (varying in the data given from 5.3 to 9.8) regardless of whether the starter had been carried at that temperature for a few or a considerable number of generations. At 20°C. on the other hand, the volatile acid production was high (varying in the data given from 32.6 to 39.3) as would be expected since this temperature represents approximately the temperature at which starters are ordinarily carried in practice. Table V gives data obtained with two starters carried at both 37°C. and 20°C. where the volatile acid determinations were run at regular intervals; from the results it is evident that with each starter the first generation at 37°C. showed only a low volatile acidity and that, after falling off, it remained fairly constant in the succeeding generations while the controls at 20°C. constantly showed a high volatile acid production. The sudden drop in the volatile acidity when a starter was carried at 37°C. is also evident in the data presented in table VI.

TABLE V. INFLUENCE OF TEMPERATURE ON VOLATILE PRODUCTION BY STARTERS.

Starter X					Starter Y				
	37°C.		20°C.			37°C.		20°C.	
Generation	Total Acidity	Volatile Acidity	Total Acidity	Volatile Acidity	Generation	Total Acidity	Volatile Acidity	Total Acidity	Volatile Acidity
1	.79	8.5	1.15	35.0	1	.73	6.5	1.01	30.0
2	.77	7.1	1.14	33.5	4	.68	7.2	1.13	37.3
3	.77	6.7	1.05	34.0	7	.79	5.7	1.12	33.1
4	.73	9.0	1.07	38.4	10	.74	6.6	.99	28.1
5	.79	7.5	1.04	36.2	13	.81	6.2	1.06	35.7
6	.78	8.8	1.04	34.5	16	.86	5.9	1.08	31.0
9	.80	7.7	----	----	19	.80	7.7	1.11	33.3
10	.72	7.6	----	----	----	----	----	----	----

One case was observed in which there was not a sudden drop in the volatile acid production when a starter was carried at 37°C. and here the volatile acidity was low in the second generation at the higher temperature and continued low thruout the four succeeding generations studied. The detailed results are given in table VII.

TABLE VI. INFLUENCE OF TEMPERATURE ON THE VOLATILE ACID PRODUCTION BY STARTERS.

	Total Acidity	Volatile Acidity
8 generations at 20°C. then 1 generation at 37°C.	.85	7.1
9 generations at 20°C. then 1 generation at 37°C.	.90	7.4
4 generations at 16°C. then 2 generations at 37°C.	.86	9.4
4 generations at 16°C. then 2 generations at 37°C.	.88	9.1

TABLE VII. INFLUENCE OF TEMPERATURE ON THE VOLATILE ACID PRODUCTION BY A STARTER.

	Total Acidity	Volatile Acidity
3 generations at 20°C. + 1 generation at 37°C.	.74	21.0
3 generations at 20°C. + 2 generations at 37°C.	.73	7.5
3 generations at 20°C. + 3 generations at 37°C.	.65	7.6
3 generations at 20°C. + 4 generations at 37°C.	.66	9.5
3 generations at 20°C. + 5 generations at 37°C.	.68	8.5
3 generations at 20°C. + 6 generations at 37°C.	.75	6.7

TABLE VIII. EFFECT OF CARRYING STARTERS AT 37°C. ON THE VOLATILE ACID PRODUCTION AT 20°C.

	Total Acidity	Volatile Acidity
7 generations at 37°C. then 1 at 20°C.	.88	7.0
15 generations at 37°C. then 1 at 20°C.	.90	6.9
9 generations at 37°C. then 1 at 20°C.	.91	7.6
3 generations at 37°C. then 1 at 20°C.	.89	8.3
3 generations at 37°C. then 2 at 20°C.	.93	6.5
3 generations at 37°C. then 3 at 20°C.	.90	5.4

TABLE IX. EFFECT OF CARRYING STARTERS AT 37°C. ON THE VOLATILE ACID PRODUCTION AT 20°C.

	Total Acidity	Volatile Acidity
1 generation at 37°C. + 3 generations at 20°C.	1.02	6.5
1 generation at 37°C. + 6 generations at 20°C.	1.04	6.2
1 generation at 37°C. + 9 generations at 20°C.	.97	6.1
1 generation at 37°C. + 12 generations at 20°C.	.97	12.3
1 generation at 37°C. + 15 generations at 20°C.	.97	7.6
1 generation at 37°C. + 18 generations at 20°C.	.97	6.0

The data which are presented in table VIII were collected with the idea of determining the effect of carrying a starter at 37°C. on the volatile acid production at 20°C. From the results it is evident that after carrying a starter for a number of generations at 37°C. and returning to 20°C., only a low volatile acidity (varying in the data given from 5.4 to 8.3) was produced. The results given in table IX, which were secured by growing a starter for one generation at 37°C. and running thru a considerable number of generations at 20°C., indicate that one generation at 37°C. is sufficient for a starter to lose its ability to produce a high volatile acidity even when returned to 20°C., and also that the ability to produce a high volatile acidity is not recovered at 20°C., altho the starter may have been held there for a considerable number of transfers.

Tables IV, V, VI, VII, VII, and IX show the general relationship between a high total acidity and a high volatile acidity that has already been pointed out, altho the low volatile acidities recorded in table IX were accompanied by higher total acidities than most of those reported in the other tables.

CONCLUSIONS AND ASSUMPTIONS FROM THE DATA PRESENTED.

The data which have been presented justify certain conclusions and assumptions regarding the starters studied and the organisms present in them.

1. Good starters from various sources that were well ripened had volatile acidities, approximating 35. Milk soured by inoculating a culture picked from an agar plate poured from a starter or secured by highly diluting a starter, ordinarily showed a much lower volatile acidity (generally under 10) which approximates the values reported by Evans for *S. lacticus*. In general a high volatile acidity was accompanied by a high total acidity.

2. There are a number of possible explanations of the situation outlined above.

a. There may be two types of *Bact. lactis acidi,* one producing a high and one a low volatile acidity. If this is the case, the data obtained suggest that the high volatile acid-producing type is present in smaller numbers than the other and either grows very slowly or not at all at 37°C.

b. The growth of the organisms on agar may change the volatile acid production. This does not seem probable since high dilutions of starters also gave a low volatile acid production.

c. There may be some organism other than *Bact. lactis acidi* that produces a high volatile acidity in a starter. The data secured indicate that if this is the case, the organism is present in smaller numbers than *Bact. lactis acidi* and grows very slowly or not at all at 37°C.

d. Mixtures of two or more cultures of *Bact. lactis acidi* may give higher volatile acidities than any of the cultures growing alone.

e. There may be an organism present in starters that influences the volatile acid production of *Bact. lactis acidi.* If this is the case, the data secured indicate that this organism is present in smaller numbers than is *Bact. lactis acidi* and grows very slowly or not at all at 37°C.

ATTEMPTS TO ISOLATE A CULTURE OF *BACT. LACTIS ACIDI* PRODUCING A HIGH VOLATILE ACIDITY.

On the assumption that there are two types of *Bact. lactis acidi,* only one of which produces a high volatile acidity, various methods were used in an attempt to isolate an organism of this type.

Direct plating of a starter was tried repeatedly. It was early observed that different media gave a very different number of colonies when the same amount of a given dilution water was used with each. This is illustrated by the following figures which were secured on plates poured with the same volume of a good starter and incubated five days at 20°C.

Medium	No. of Colonies
Beef infusion agar	70
Lactose beef extract agar	78
Beerwort agar	213
Whey agar	311

Many organisms in addition to those reported in table II were isolated from plates poured with various media and tested for volatile acid production. In general only low volatile acidities were secured; the very few exceptions to this situation will be considered later.

Pieces of agar were picked from between colonies and put into tubes of sterile milk in the belief that the lactic acid organisms causing the high acidity were not growing on the media used; growth was rarely secured in the tubes but the organisms present were like those secured from colonies in that they produced only a low volatile acidity and gave a growth when inoculated onto an agar slope, and accordingly it seems that growth had simply been delayed on the agar plate.

An attempt was made to secure a lactic acid organism producing a high volatile acidity by the use of heat, temperatures from 53° to 60°C. being used for 5 and 10-minute periods after a small amount of starter had been inoculated into sterile milk. Usually a low volatile acidity was secured when the tubes of heated milk which had given growth were used for inoculating sterile milk. In one instance under the above conditions a high volatile acidity (31.7) was secured; however, when the material was plated out

TABLE X. VOLATILE ACID PRODUCTION OF STARTERS AFTER SEVERAL GENERATIONS UNDER AN ANTISEPTIC.

	Total Acidity	Volatile Acidity
6 generations under toluol	.76	6.0
5 generations under toluol	.82	8.7
7 generations under ether	.92	8.2

the usual type of colony producing only a low volatile acidity was obtained. From the work carried out it seems improbable that a lactic acid organism producing a high volatile acidity can be secured as a result of resistance to heat.

By adding toluol or ether to a tube of milk into which a small amount of starter has been inoculated and shaking lightly, a coagulation can commonly be obtained after incubation. Sometimes a starter can be run thru a number of generations under toluol or ether if the antiseptic is not too thoroly distributed thru the milk, altho in other cases it may be impossible to get even the first inoculation to grow. It was thought that toluol or ether might exert a selective action so that a high volatile acid-producing lactic acid organism could be secured as a result of the elimination of the low volatile acid producers. The results obtained in such an attempt are presented in table X; they show that after a number of generations under an antiseptic only a low volatile acid production was secured.

Because starters carried at temperatures fairly high for *Bact. lactis acidi* (e. g. 37°C.) gave only a low volatile acid production, it was thought that low temperatures might be an aid in the securing of a lactic acid organism producing a high volatile acidity. Accordingly, starters were run thru a number of transfers at from 12° to 16°C., the temperature being maintained by means of Dewar flasks. After such transfers the starters were plated but only low volatile acid-producing lactic acid organisms were secured. The dilution method was also used but the tubes which were inoculated with the smaller amounts of material and gave growth, produced only a low volatile acidity when grown in milk at 20°C. Some of the data secured with the dilution method are given in table XI.

Since a high volatile acidity was ordinarily accompanied by a high total acidity, an attempt was made to isolate an organism producing a high volatile acidity by allowing a starter to stand and making transfers from it from time to time with the idea that the last transfer to give a good growth would contain only a lactic acid organism producing a high volatile acidity. Starter 18 was put at approximately 20°C. on April 18th and transfers made from it every few days; one of the last transfers from it to give a

TABLE XI. VOLATILE ACID PRODUCTION BY VARIOUS DILUTIONS OF STARTERS.

Dilution of starter after 2 generations at 12–16°C.				Dilution of starter after 2 generations at 12–16°C.			
Tube No.	Incubation of Milk Distilled	Total Acidity	Volatile Acidity	Tube No.	Incubation of Milk Distilled	Total Acidity	Volatile Acidity
8	3 days, 20°C.	1.06	32.0	8	6 days, 20°C.	1.06	41.4
12	3 days, 20°C.	.93	13.6	9	3 days, 20°C.	1.11	36.8
13	3 days, 20°C.	.86	8.6	10	7 days, 20°C.	.99	7.3
				11	7 days, 20°C.	.95	7.1

TABLE XII. VOLATILE ACID PRODUCTION BY CULTURES SECURED FROM PLATES POURED FROM TRANSFER GIVING HIGH VOLATILE ACIDITY.

Culture	Incubation of Milk Distilled	Total Acidity	Volatile Acidity
18-1	7 days, 20°C.	.97	6.5
18-1	6 days, 37°C.	.64	8.1
18-2	3 days, 20°C.	1.12	36.7
18-2	5 days, 20°C.	1.10	34.5
18-2	7 days, 20°C.	1.15	41.6
18-3	4 days, 20°C.	1.05	36.7

TABLE XIII. VOLATILE ACID PRODUCTION OF CULTURES SECURED BY PLATING CULTURE 18-2.
(A High Volatile Acid Producing Culture.)

Culture	Incubation of Milk Distilled	Total Acidity	Volatile Acidity
18-2 rep. 1	4 days, 20°C.	1.12	40.8
18-2 rep. 2	3 days, 20°C.	.97	7.5

TABLE XIV. VOLATILE ACID PRODUCTION OF CULTURES SECURED BY PLATING CULTURE 18-2 REP. 1.
(A High Volatile Acid Producing Culture.)

Culture	Incubation of Milk Distilled	Total Acidity	Volatile Acidity
18-2, rep. 1, rep. 1	7 days, 20°C.	.95	8.8
18-2, rep. 1, rep. 2	6 days, 20°C.	.93	10.6
18-2, rep. 1, rep. 3	7 days, 20°C.	.91	7.5
18-2, rep. 1, rep. 4	6 days, 20°C.	.86	7.2
18-2, rep. 1, rep. 5	7 days, 20°C.	.97	10.1
18-2, rep. 1, rep. 6	7 days, 20°C.	.90	8.3

rapid coagulation was made on May 7th and this was studied in some detail. At 20°C. the original trial showed a volatile acid production of 33.8 (total acidity 1.09) and this high volatile acid production was maintained during the time the transfer was studied. In order to test the purity of the transfer it was plated out, the plates held at 20°C. and colonies were then picked into milk. Out of three colonies studied two produced a high volatile acidity and one produced a low volatile acidity. Altho many tests were made these three colonies persisted in the original type of volatile acid production; a few of the values secured are presented in table XII.

Culture 18-2, which was one of the two yielding a high volatile acidity was plated out and two colonies picked from the plates. The volatile acid production of these two colonies at 20°C. is given in table XIII.

Table XIII shows that of the two colonies picked one was a high and one a low volatile acid-producing culture. Each of these cultures was then plated out, colonies picked, and the volatile acid production studied; the results secured are presented in tables XIV and XV.

From tables XIV and XV, it is evident that the cultures (six) picked from a plate poured from a high volatile acid-producing culture produced only low volatile acidities as did also the cultures (six) picked from a plate poured from a low volatile acid-producing culture. The data presented in tables XIII, XIV and XV indicate that while an occasional colony yielding a high volatile acidity may be picked from an agar plate, further plating will yield low volatile acid-producing cultures either entirely or in part.

While various procedures were being tried out in an attempt to isolate a high volatile acid-producing lactic acid organism, starters were occasionally plated direct, colonies picked, and the volatile acidity produced in milk by the cultures determined. Shortly after the first colonies giving a high volatile acidity were picked from agar plates poured from an old transfer from a starter (as

TABLE XV. VOLATILE ACID PRODUCTION OF CULTURES SECURED BY PLATING CULTURE 18-2 REP. 2.
(A Low Volatile Acid Producing Culture.)

Culture	Incubation of Milk Distilled	Total Acidity	Volatile Acidity
18-2, rep. 2, rep. 1	6 days, 20°C.	.95	11.6
18-2, rep. 2, rep. 2	6 days, 20°C.	.98	15.6
18-2, rep. 2, rep. 3	6 days, 20°C.	.90	12.6
18-2, rep. 2, rep. 4	6 days, 20°C.	.97	13.6
18-2, rep. 2, rep. 5	6 days, 20°C.	.94	12.0
18-2, rep. 2, rep. 6	6 days, 20°C.	.95	10.2

outlined above), colonies of this type were also secured by plating a starter direct. The volatile acidities produced in the original trials with seven cultures picked from one plate are shown in table XVI.

From table XVI, it will be seen that out of the seven colonies picked, four gave a high volatile acidity when grown in milk while three did not. Altho starters had been plated out many times previously, the colonies referred to in table XVI were the first ones yielding a high volatile acidity that were obtained by direct plating, and in subsequent attempts such colonies were only very rarely secured. An explanation of these results is offered later.

Culture A1 which table XVI shows to be a high volatile acid-producing culture, was plated out and seven colonies picked; the volatile acid production of these colonies is shown in table XVII.

From table XVII it will be seen that of the seven cultures studied only one yielded a high volatile acidity; accordingly it is evident that the cultures obtained by direct plating which yield a high volatile acidity also fail to maintain their volatile acid production on subsequent plating.

From the results presented in tables XII-XVII, inclusive, it is evident that the very few high volatile acid-producing cultures secured by picking colonies from plates failed to yield only high volatile acid-producing cultures when again plated as they would

TABLE XVI. VOLATILE ACID PRODUCTION OF CULTURES PICKED FROM AN AGAR PLATE POURED FROM A STARTER.

Culture	Incubation of Milk Distilled	Total Acidity	Volatile Acidity
A1	3 days, 20°C.	1.11	43.0
A2	3 days, 20°C.	.90	10.0
A3	3 days, 20°C.	1.03	31.5
A4	4 days, 20°C.	.90	6.8
A5	4 days, 20°C.	.99	36.5
A6	3 days, 20°C.	.99	40.6
A7	4 days, 20°C.	.90	6.1

TABLE XVII. VOLATILE ACID PRODUCTION OF CULTURES SECURED BY PLATING CULTURE A1.
(A High Volatile Acid Producing Culture.)

Culture	Incubation of Milk Distilled	Total Acidity	Volatile Acidity
A1, rep. 1	5 days, 20°C.	.87	4.8
A1, rep. 2	5 days, 20°C.	1.04	42.7
A1, rep. 3	5 days, 20°C.	.90	9.2
A1, rep. 4	3 days, 20°C.	.94	6.9
A1, rep. 5	5 days, 20°C.	.83	6.0
A1, rep. 6	5 days, 20°C.	.91	7.7
A1, rep. 7	3 days, 20°C.	.77	6.3

be expected to do if they were pure; they were in fact more likely to yield low volatile acid-producing colonies. This suggests strongly that the cultures yielding a high volatile acidity were impure and this idea is supported by the observation that the colonies giving a high volatile acidity were in general picked from plates that were somewhat heavily seeded and which were accordingly more likely to yield impure culture, as well as by data reported later. Accordingly, it seems justifiable to conclude that it is impossible to isolate a pure culture of *Bact. lactis acidi* that produces a volatile acidity at all comparable with the volatile acidity produced by a good starter.

INFLUENCE ON VOLATILE ACID PRODUCTION OF GROWING THE STARTER ORGANISMS ON AGAR.

In order to study the influence on the volatile acid production of growing the starter organisms on agar a good starter was selected and a very small amount transferred to a whey agar slope with a loop; when growth was evident, a transfer was made to another whey agar slope and this was repeated thru a number of transfers. Some of the transfers were inoculated into milk and after growth occurred, volatile acid determinations were made. The same procedure was followed with a number of starters and the results secured are presented in table XVIII.

From table XVIII, it is evident that a starter can be run thru a number of transfers on whey agar (at least 12 transfers in two instances) without losing its ability to produce a high volatile acidity. This indicates that the growth on the agar in the plates was not responsible for the failure to isolate high volatile acid-producing organisms. The same idea is suggested by the fact that in general only low volatile acid-producing organisms were secured when sterile milk in considerable quantities (1 c.c.) was added, at the time of pouring, to the plates from which the colonies were picked since it would be expected that it would be the lack of milk rather than the presence of any of the agar constituents that would be responsible for any possible effect.

A starter cannot be grown indefinitely on agar and retain its

TABLE XVIII. VOLATILE ACID PRODUCTION OF VARIOUS STARTERS AFTER A NUMBER OF GENERATIONS ON AGAR.

Starter	Generations on Agar	Total Acidity	Volatile Acidity
A	1	.99	39.4
	8	1.13	41.2
B	4	1.05	36.9
C	4	1.11	32.2
	12	1.10	39.2
D	4	1.05	33.9
E	4	.99	37.2
	12	.95	49.3

usual character, as will be shown later; this is not due to any special effect of the agar, as evident from the data presented.

One of the points that should be considered in connection with the effect of a number of transfers on agar on the volatile acid production is whether or not a certain volatile acid production, either high or low, is constant with a given culture over a somewhat extended period of time and many transfers in milk. A considerable amount of evidence on this question has been accumulated in an indirect way so that a record of the number of transfers or similar information is not available. Both high and low volatile acid-producing cultures have been run at widely separated times and the original type of volatile acid production found has been maintained. The constancy of a high volatile acid production with a good starter is additional evidence on this point. A certain type of volatile acid production (either high or low) was also maintained, when a number of transfers were held for considerable periods with the original cultures at the temperature existing in a refrigerated room. For more definite evidence, however, two low volatile acid-producing organisms were selected, transferred frequently in milk at 20°C., and a record kept of the number of transfers. At various times the organisms were each inoculated into milk and the volatile acid production determined, with the results presented in table XIX. These data show that in both cases an organism which gave a low volatile acidity when first picked from an agar plate maintained this type of volatile acid production after 47 transfers in milk at 20°C.

ATTEMPTS TO ISOLATE AN ORGANISM OTHER THAN *BACT. LACTIS ACIDI* PRODUCING A HIGH VOLATILE ACIDITY.

When plates were poured from good starters, organisms other than the typical *Bact. lactis acidi* type, some of which would not coagulate milk, were occasionally isolated. Altho they never occurred in large numbers, certain of these organisms were tried out for high volatile acid production both alone and in various combinations, but always with negative results. *Bact. aerogenes* has been reported[6] as fermenting the citric acid in milk with the formation of acetic acid and CO_2 and accordingly three cultures of this organism that were being carried with the laboratory stock cultures were tried out for their volatile acid production in milk, altho it does not seem at all probable that this organism would have been missed if it were present in a starter since it grows so well on ordinary media; the results obtained are given in table XX.

From table XX it is evident that the volatile acidity produced by the cultures of *Bact. aerogenes* studied was low when compared to the volatile acidity produced by good starters and that

TABLE XIX. VOLATILE ACID PRODUCTION OF TWO ORGANISMS AFTER VARIOUS NUMBERS OF TRANSFERS AT 20°C.

Culture A				Culture B			
Transfers in milk	Incubation of milk distilled	Total acidity	Volatile acidity	Transfers in milk	Incubation of milk distilled	Total acidity	Volatile acidity
2	3 days, 20°C.	.94	11.9	2	5 days, 20°C.	.99	6.9
20	3 days, 20°C.	.91	5.5	20	3 days, 20°C.	.93	6.2
21	7 days, 20°C.	.92	5.3	21	7 days, 20°C.	.90	7.5
46	7 days, 20°C.	----------	6.3	46	7 days, 20°C.	----------	7.5
47	8 days, 20°C.	.92	4.8	47	8 days, 20°C.	.81	6.0

[6]Bosworth, Alfred W., and Prucha, M. J. The Fermentation of Citric Acid in Milk. N. Y. Agr. Expt. Sta. Tech. Bul. 14. 1910.

TABLE XX. VOLATILE ACID PRODUCTION BY BACT. AEROGENES.

Culture No.	Total Acidity	Volatile Acidity
1	.81	12.0
2	.90	13.2
3	.65	9.7

accordingly this organism is not of importance in producing the high volatile acidity in starters.

While the results presented are not extensive they indicate that the volatile acidity of good starters is not due to some organism other than *Bact. lactis acidi.*

EFFECT ON THE VOLATILE ACID PRODUCTION OF MIXING CULTURES OF *BACT. LACTIS ACIDI.*

In an effort to explain the high volatile acid production of starters, mixtures of various cultures of *Bact. lactis acidi,* each producing a low volatile acidity, were tried. In the early trials the volatile acidities were determined only on the first milk fermented after mixing two organisms. When these attempts yielded only low volatile acidities, the mixtures were run thru a number of transfers and the volatile acid production of each transfer of each combination determined. One set of data secured with this method is shown in table XXI; from this it is evident that six different combinations, each containing two organisms producing a low volatile acidity, failed to produce a high volatile acidity during eight transfers after the mixing. Each of the organisms used in combination was carried alone as was also one high volatile acid-producing culture (presumably impure), and the original type of volatile acid production was maintained; this is additional evidence of the constancy of a culture in the type of its volatile acid production. Similar results were secured in other trials, using mixtures of two organisms.

When two cultures, each producing a low volatile acidity, failed to develop a high volatile acidity when grown in combination, mixtures of more than two of the organisms were tried. Some of the data secured are presented in table XXII; from these results it is evident that the more complex combinations of organisms used failed to give a high volatile acidity even after a number of transfers.

The results presented indicate that the high volatile acid production of starters is not the result of mixtures of two or more cultures of *Bact. lactis acidi.*

PRESENCE IN STARTERS OF ORGANISMS WHICH INFLUENCE VOLATILE ACID PRODUCTION OF *BACT. LACTIS ACIDI.*

When colonies yielding a high volatile acidity were first picked from plates and before the data obtained indicated that they were

TABLE XXI. VOLATILE ACID PRODUCTION OF *BACT. LACTIS ACIDI* ORGANISMS ALONE AND IN COMBINATION DURING A NUMBER OF TRANSFERS.

Transfer No.	Org. 1			Org. 2			Org. 5			Org. 6			Org. 7		
	Days at 20°	Total Acidity	Volatile Acidity	Days at 20°	Total Acidity	Volatile Acidity	Days at 20°	Total Acidity	Volatile Acidity	Days at 20°	Total Acidity	Volatile Acidity	Days at 20°	Total Acidity	Volatile Acidity
1	4	.74	5.1	5	.91	49.2	4	.85	7.1	4	.80	8.3	4	.78	6.9
2	6	.77	6.4	6	.87	47.3	6	.82	6.3	6	.82	8.2	6	.77	6.1
3	3	.71	5.9	3	.91	37.9	3	.77	5.5	3	.77	4.2	3	.74	6.1
4	4	.85	5.4	5	1.01	46.1	4	.86	6.6	4	.89	9.7	4	.88	7.0
5	3	------	8.0	2	.66	33.6	3	.64	6.8	3	------	9.9	4	.56	7.5
6	5	.82	5.4	4	.84	38.1	4	.85	4.6	4	.85	9.9	3	.81	6.5
7	8	.84	5.1	8	1.02	38.0	8	.86	5.7	8	.86	5.1	8	.90	7.4
8	6	.86	5.0	7	.97	40.5	7	.86	7.7	7	.89	4.0	7	.87	5.1

Transfer No.	Org. 1 plus Org. 5			Org. 1 plus Org. 6			Org. 1 plus Org. 7			Org. 5 plus Org. 6			Org. 5 plus Org. 7			Org. 6 plus Org. 7		
	Days at 20°	Total Acidity	Volatile Acidity	Days at 20°	Total Acidity	Volatile Acidity	Days at 20°	Total Acidity	Volatile Acidity	Days at 20°	Total Acidity	Volatile Acidity	Days at 20°	Total Acidity	Volatile Acidity	Days at 20°	Total Acidity	Volatile Acidity
1	4	.82	5.7	5	.79	6.1	4	.77	5.5	4	.80	6.1	4	.82	6.4	5	.82	6.0
2	6	.84	6.4	6	.85	7.5	6	.86	7.4	6	.82	7.5	6	.83	7.1	6	.82	6.7
3	3	.72	4.4	3	.81	6.2	3	.78	6.1	3	.79	4.7	3	.76	-----	4	.77	6.3
4	4	.92	7.3	4	.90	6.1	4	.86	5.6	4	.89	6.4	4	.86	5.8	4	.81	6.1
5	3	.63	7.5	3	.54	9.0	3	.64	8.0	3	.63	8.6	3	.56	7.5	3	.59	9.0
6	4	.85	5.7	5	.81	5.0	4	.81	5.7	4	.83	7.5	5	.82	6.0	4	.79	9.5
7	8	.86	5.6	8	.81	5.5	8	.85	8.1	8	.86	4.5	10	.79	-----	7	.87	9.9
8	7	.88	4.6	7	.85	4.8	7	.88	6.1	7	.90	4.7	7	.88	4.7	7	.79	7.7

impure, rabbits were immunized with these cultures and also with low volatile acid-producing cultures for the purpose of studying the agglutinin production. The organisms used for immunization were grown on whey agar slopes; after a number of transfers the cultures which originally produced a high volatile acidity failed to curdle milk when transferred back to it, altho growth was maintained on the whey agar slopes. Ordinary starter smeared on a whey agar slope and then transferred for a number of times on such slopes also finally gave a growth which failed to curdle milk into which it was inoculated, altho, as has already been shown, the high volatile acid production of starters was maintained on whey agar slopes for a number of transfers. Cultures of *Bact. lactis acidi* which produced the usual low volatile acidity retained their ability to curdle milk during the many transfers on whey agar that were made in an attempt to have them lose this property. All of the whey agar slope cultures which failed to curdle milk had an appearance much like that of a culture of *Bact. lactis acidi* altho they were somewhat heavier than a typical growth of this organism. These results suggested that there might be associated with *Bact. lactis acidi* in a good starter and influencing its volatile acid production, an organism which would not curdle milk alone, and which could be quite

TABLE XXII. VOLATILE ACID PRODUCTION THRU A NUMBER OF TRANSFERS OF MIXTURES OF *BACT. LACTIS ACIDI* CULTURES EACH PRODUCING A LOW VOLATILE ACIDITY.

Mixture A			Mixture B			Mixture C		
10 Cultures from 1 Plate			10 Cultures from 1 Plate			12 Cultures from 1 Plate		
Transfer After Mixing	Total Acidity	Volatile Acidity	Transfer After Mixing	Total Acidity	Volatile Acidity	Transfer After Mixing	Total Acidity	Volatile Acidity
1	.83	9.9	1	.87	9.7	1	.93	7.8
5	.98	5.0	5	.83	7.0	2	.98	5.5
6	.85	3.8	6	.87	4.1	3	-----	6.5
7	.98	7.7	7	.95	8.3	4	-----	8.0
8	-----	6.4	8	-----	7.2	5	.96	4.9
9	.81	4.0	9	.81	4.0	10	.94	3.6
10	.90	7.0	10	.91	6.8	-------	-----	-----

readily secured by growing a starter on whey agar for a number of transfers in order to gradually eliminate *Bact. lactis acidi.* This idea was tested out by comparing the volatile acid production of *Bact. lactis acidi* alone, with the volatile acid production of *Bact. lactis acidi* in combination with a culture which failed to curdle milk. Some of the data secured in the early trials are presented in table XXIII.

From table XXIII, it is evident that the volatile acid production of *Bact. lactis acidi* alone was much less than the volatile acid production of *Bact. lactis acidi* grown in combination with another organism (obtained from starter), which alone did not produce coagulation in milk. The volatile acid production when the organisms were combined was essentially the same as the volatile acid production of good starters.

After the results presented in table XXIII were secured an attempt was made to isolate the organism associated with *Bact. lactis acidi* directly from starter. In the isolation of organisms from plates poured from starters it was a common experience to find some of the tubes of litmus milk into which colonies had been inoculated, unchanged even after extended periods of incubation, and these tubes had been assumed to represent cases in which the desired colony had been missed. Some of these old tubes of litmus milk were cultured on whey agar slopes and in certain cases colonies developed. With this information as a basis the following method was tried for the securing of the associated organism.

Plates were poured from a starter, usually with whey agar; about 1 c.c. of sterile milk was sometimes added to the plates since the milk seemed to hasten the development of the colonies. After incubating the plates at 20°C. a number of colonies were streaked on whey agar slopes and after a good growth had developed a tube of litmus milk was inoculated from each slope.

TABLE XXIII. VOLATILE ACID PRODUCTION OF *BACT. LACTIS ACIDI* ALONE IN COMPARISON WITH THAT OF *BACT. LACTIS ACIDI* IN COMBINATION WITH ANOTHER ORGANISM ISOLATED FROM STARTER.

Material Used for Inoculating	Total Acidity	Volatile Acidity
Bact. lactis acidi 322	.87	5.7
Bact. lactis acidi 322 + x* 15 transfers on whey agar	1.00	36.9
Bact. lactis acidi 325	.95	5.2
Bact. lactis acidi 325 + x 15 transfers on whey agar	1.07	32.4
Bact. lactis acidi 321	.91	5.8
Bact. lactis acidi 321 + x 17 transfers on whey agar	.96	31.9
Bact. lactis acidi 321 + y** 27 transfers on whey agar	1.06	38.5
Bact. lactis acidi a	.90	5.2
Bact. lactis acidi a + x 17 transfers on whey agar	1.14	36.2
Bact. lactis acidi a + y 27 transfers on whey agar	1.13	36.2

*x—Growth secured by transferring starter to a whey agar slope.

**y—Growth secured by transferring to a whey agar slope milk curdled by inoculating with a colony that was supposedly impure since it gave a high volatile acidity.

TABLE XXIV. VOLATILE ACID PRODUCTION OF *BACT. LACTIS ACIDI* ALONE IN COMPARISON WITH THAT OF *BACT. LACTIS ACIDI* IN COMBINATION WITH THE ASSOCIATED ORGANISM FROM THE SAME STARTER. USING 1 *BACT. LACTIS ACIDI* ISOLATION AND 7 ASSOCIATED ORGANISM ISOLATIONS.

Material used for Inoculating	Total Acidity	Volatile Acidity
Bact. lactis acidi alone	.86	6.5
Bact. lactis acidi + Associated Org. 1	1.10	33.7
Bact. lactis acidi + Associated Org. 2	1.10	33.3
Bact. lactis acidi + Associated Org. 3	.97	38.2
Bact. lactis acidi + Associated Org. 4	1.12	39.7
Bact. lactis acidi + Associated Org. 5	1.14	37.8
Bact. lactis acidi + Associated Org. 6	1.12	34.0
Bact. lactis acidi + Associated Org. 7	1.07	38.5

The colonies that gave a good growth on a whey agar slope and failed to curdle milk were considered to represent the associated organism and were tried out for their influence on the total acid and volatile acid production of *Bact. lactis acidi*. A portion of the data obtained with this method sufficient to show the general trend of the results is presented in tables XXIV, XXV and XXVI. From these tables it is evident that the mixtures of *Bact. lactis acidi* and the associated organism gave a higher volatile acidity than *Bact. lactis acidi* alone, and also a higher total acidity.

With the results presented in tables XXIII, XXIV, XXV, and XXVI, no attention was given to the volatile acid production of the associated organism alone, so additional results were secured, taking this point into consideration. Some of the data obtained are given in tables XXVII, XXVIII and XXIX.

From tables XXVII, XXVIII, and XXIX, it is evident that the volatile acid production of the associated organism alone is greater than the volatile acid production of *Bact. lactis acidi*. In the tables mentioned the volatile acidity of the former organ-

TABLE XXV. VOLATILE ACID PRODUCTION OF *BACT. LACTIS ACIDI* ALONE IN COMPARISON WITH THAT OF *BACT. LACTIS ACIDI* IN COMBINATION WITH THE ASSOCIATED ORGANISM FROM THE SAME STARTER. USING 5 *BACT. LACTIS ACIDI* ISOLATIONS AND 1 ASSOCIATED ORGANISM ISOLATION.

Material used for Inoculating	Total Acidity	Volatile Acidity
Bact. lactis acidi 1	.90	8.0
Bact. lactis acidi 1 + Associated Org. 1	1.08	33.7
Bact. lactis acidi 2	.90	7.5
Bact. lactis acidi 2 + Associated Org. 1	1.11	36.7
Bact. lactis acidi 3	.90	8.4
Bact. lactis acidi 3 + Associated Org. 1	1.08	35.5
Bact. lactis acidi 4	.91	6.7
Bact. lactis acidi 4 + Associated Org. 1	.94	35.8
Bact. lactis acidi 5	.93	7.0
Bact. lactis acidi 5 + Associated Org. 1	1.11	39.0

TABLE XXVI. VOLATILE PRODUCTION OF *BACT. LACTIS ACIDI* ALONE IN COMPARISON WITH THAT OF *BACT. LACTIS ACIDI* IN COMBINATION WITH THE ASSOCIATED ORGANISM FROM THE SAME STARTER. USING 3 *BACT. LACTIS ACIDI* ISOLATIONS AND 2 ASSOCIATED ORGANISM ISOLATIONS.

Material used for Inoculating	Total Acidity	Volatile Acidity
Bact. lactis acidi 1	.76	4.2
Bact. lactis acidi 1 + Associated Org. 1	.82	31.7
Bact. lactis acidi 1 + Associated Org. 2	.78	31.0
Bact. lactis acidi 2	.73	5.1
Bact. lactis acidi 2 + Associated Org. 1	.79	30.0
Bact. lactis acidi 2 + Associated Org. 2	.77	31.8
Bact. lactis acidi 3	.73	4.2
Bact. lactis acidi 3 + Associated Org. 1	.85	31.5
Bact. lactis acidi 3 + Associated Org. 2	.81	31.2

TABLE XXVII. VOLATILE ACID PRODUCTION OF *BACT. LACTIS ACIDI* ALONE, THE ASSOCIATED ORGANISM ALONE, AND THE TWO IN COMBINATION. USING 3 *BACT. LACTIS ACIDI* ISOLATIONS AND 3 ASSOCIATED ORGANISM ISOLATIONS.

Material Used for Inoculating	Total Acidity	Volatile Acidity
Bact. lactis acidi 1	.73	4.5
Bact. lactis acidi 2	--------	6.3
Bact. lactis acidi 3	.81	7.5
Associated Org. 1	.26	18.1
Associated Org. 2	.31	15.9
Associated Org. 3	.24	16.0
Bact. lactis acidi 1 + Associated Org. 1	.83	32.2
Bact. lactis acidi 1 + Associated Org. 2	.87	33.1
Bact. lactis acidi 1 + Associated Org. 3	.78	29.5
Bact. lactis acidi 2 + Associated Org. 1	.88	37.5
Bact. lactis acidi 2 + Associated Org. 2	.92	39.2
Bact. lactis acidi 2 + Associated Org. 3	.89	28.7
Bact. lactis acidi 3 + Associated Org. 2	.90	35.0
Bact. lactis acidi 3 + Associated Org. 3	.90	33.2

TABLE XXVIII. VOLATILE ACID PRODUCTION OF *BACT. LACTIS ACIDI* ALONE, THE ASSOCIATED ORGANISM ALONE, AND THE TWO IN COMBINATION. USING 3 *BACT. LACTIS ACIDI* ISOLATIONS AND 2 ASSOCIATED ORGANISM ISOLATIONS.

Material Used for Inoculating	Total Acidity	Volatile Acidity
Bact. lactis acidi 1	.91	4.3
Bact. lactis acidi 2	.87	4.6
Bact. lactis acidi 3	.91	4.8
Associated Organism 1	.25	14.3
Associated Organism 2	.25	14.2
Bact. lactis acidi 1 + Associated Org. 1	1.01	33.5
Bact. lactis acidi 1 + Associated Org. 2	1.08	36.5
Bact. lactis acidi 2 + Associated Org. 1	1.12	31.5
Bact. lactis acidi 2 + Associated Org. 2	1.10	36.4
Bact. lactis acidi 3 + Associated Org. 1	.99	36.2
Bact. lactis acidi 3 + Associated Org. 2	1.04	34.0

TABLE XXIX. VOLATILE ACID PRODUCTION OF *BACT. LACTIS ACIDI* ALONE, THE ASSOCIATED ORGANISM ALONE, AND THE TWO IN COMBINATION. USING 2 *BACT. LACTIS ACIDI* ISOLATIONS AND 2 ASSOCIATED ORGANISM ISOLATIONS.

Material used for Inoculating	Total Acidity	Volatile Acidity
Bact. lactis acidi 1	.81	4.2
Bact. lactis acidi 2	.86	3.8
Associated Organism 1	.26	17.8
Associated Organism 2	.26	15.8
Bact. lactis acidi 1 + Associated Org. 1	.99	28.0
Bact. lactis acidi 1 + Associated Org. 2	.98	32.1
Bact. lactis acidi 2 + Associated Org. 1	1.03	29.5
Bact. lactis acidi 2 + Associated Org. 2	1.02	33.4

ism varied from 14.2 to 18.1 while that of the latter organism varied from 3.8 to 7.5. In no instance did the volatile acidity of either organism alone or the sum of the volatile acidities produced by the two organisms alone equal the volatile acidity of the two organisms in combination which varied in the tables under discussion from 28.0 to 39.2. The increase in the total acidity produced when the two organisms were grown in combination over that produced by *Bact. lactis acidi* alone is in general evident also. On the assumption that the acidity produced limits the growth it is apparent that the associated organism has a definite effect on the total acidity tolerated by *Bact. lactis acidi.* The increase in the total acidity as a result of combining the two organisms is however by no means as great on a percentage basis as is the increase in the volatile acidity.

DISCUSSION.

In a study of the volatile acid production of starters and the organisms isolated from them it was found that the volatile acid production of *Bact. lactis acidi* was much lower than that of the starter from which it was isolated.

The figures which were secured for the volatile acid production of *Bact. lactis acidi* are in essential agreement with the results reported by Evans, who found in a study of the streptococci concerned in cheese ripening that there are other organisms that produce considerably more volatile acidity than does *S. lacticus.* The data herein presented indicate that the volatile acid production of *Bact. lactis acidi* and an organism associated with it in the starters studied was decidedly different than the volatile acid production of *Bact. lactis acidi* alone. The associated organism when grown in milk alone did not cause coagulation.

Associative action among organisms has been studied in considerable detail by Marshall[7] and his associates, particularly from the standpoint of the souring of milk. They found that the growth of lactic acid organisms may or may not be facilitated by the bacteria ordinarily found in milk. About 57% of the associate microorganisms when grown in combination with the lactic acid organisms accelerated the growth and action of the latter. The results herein reported substantiate the findings of Marshall and his associates and also show the effect of combining certain organisms on the production of volatile acidity.

The associative action of bacteria is important in fermentations other than the lactic acid fermentation. The influence of *Bact. lactis acidi* on the color production of *B. cyanogenes*[8] is a significant change in appearance due to the combined action of organisms and associative action of organisms has been reported[9] as responsible for the development of ropiness in milk. From the data presented it is evident that the production of volatile acidity is another change that is influenced markedly by associative action.

[7]Marshall, Chas. E. and Farrand, Bell. Bacterial Associations in the Souring of Milk. Mich. Agr. Expt. Sta. Spec. Bulletin 42. 1908, also preceding bulletins.

[8]Hammer, B. W. A Bacteriological Study of Blue Milk. Ia. Agr. Expt. Sta. Res. Bul. 15. 1914.

[9]Buchanan, R. E. and Hammer, B. W. Slimy and Ropy Milk, Ia. Agr. Expt. Sta. Res. Bul. 22. 1915.

It seems that in most instances of associative action one of the organisms concerned is *Bact. lactis acidi.* The *Bact. lactis acidi* group is, of course, a variable one and whether or not different strains of *Bact. lactis acidi* behave differently in combination with other organisms from the standpoint of volatile acid production cannot be definitely stated at present. In the instance above referred to, where a ropy condition resulted from the growth of *Bact. lactis acidi* and another organism, it was impossible to produce this change with all strains of *Bact. lactis acidi* studied.

There are many phases of the problem that need further investigation and a number of these are at present under study. One of the important points to determine is whether or not the associated organism from different starters represents a single type or a group; this question will be considered in an early publication along with a description of the associated organism.

SUMMARY.

1. In a number of starters of very satisfactory quality and from different sources that were studied more than one organism was present. This is evident from the following:

a. Cultures of *Bact. lactis acidi* picked from agar plates poured with starter gave a much lower volatile acidity in milk than did the starter itself.

b. By gradually diluting a starter a culture was secured which coagulated milk readily, but which yielded only a low volatile acidity that was essentially the same as the volatile acidity yielded by cultures of *Bact. lactis acidi* picked from agar plates.

c. Two organisms were isolated from starter which in combination gave a volatile acidity approximating the volatile acidity secured from the starter, altho each of these organisms alone gave a volatile acidity considerably below the volatile acidity produced by the starter.

2. The high volatile acidity of starters is not due to the action of *Bact. lactis acidi* alone.